Clinical, Diagnostic and Biochemical Features of Generalized Glycogenosis Type II in Brahman Cattle

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Summary

Clinical, diagnostic and biochemical features of generalized glycogenosis are described in 96 Brahman-type calves. Typically the calves were presented when about 6 months of age, with ill-thrift and muscular weakness as the most common signs. Acidic α-glucosidase activity was reduced in peripheral blood lymphocytes and skeletal muscle. Muscle glycogen concentration was consistently higher in affected animals than in clinically normal cattle. Other observations in affected calves included elevation of serum aspartate aminotransferase and creatine kinase activities and excessive amounts of high molecular weight oligosaccharides in urine. Fine cytoplasmic vacuolation of neurones in the brain and spinal cord, skeletal muscle, myocardium and of Purkinje fibers were consistent histological observations. Periodic acid-Schiff staining revealed the presence of glycogen-like material in peripheral blood lymphocytes of all affected calves, indicating that this is a useful aid for the diagnosis of glycogenosis. While 3 of the 96 calves showed somewhat different clinical signs, the similarity of pathology and the biochemical and clinical evidence in the remainder suggested that, in these animals, the disease was expressed as a single syndrome. *Aust Vet J* 70: 405-408

Introduction

Generalized glycogenosis (glycogen storage disease type II; Pompe's disease; 232300 [McKusick 1990]) is an autosomal recessive disease caused by a deficiency of acidic 1,4 α-glucosidase (AAG). The disease has been diagnosed in man (Pompe 1932), dog (Mostafa 1970), sheep (Manktelow and Hartley 1975), quail (Murakami *et al* 1980) and in 2 breeds of cattle in Australia (Jolly *et al* 1977; O'Sullivan *et al* 1981).

In Shorthorn cattle Howell *et al* (1981) observed 2 forms of the disease that bear similarities to variations in the presentation of generalized glycogenosis in man (Engel *et al* 1973). O'Sullivan *et al* (1981) described 6 cases of glycogenosis in Brahman calves. We present observations on clinical, biochemical and pathological aspects of 96 cases of generalized glycogenosis in Brahman calves. In addition we evaluated an alternative laboratory test for confirmation of a diagnosis of generalized glycogenosis.

Materials and Methods

Animals and Samples

Between 1981 and 1992, glycogenosis was diagnosed, on the basis of history, clinical signs and biochemical and/or histological examination of blood and/or tissue samples, in 96 Brahman or Brahman-type calves submitted by field veterinarians in Queensland. Of these, 84 animals were identified and sampled in the field, 8 were submitted to laboratories for necropsy and 4 were submitted for more intensive study. Of these last 4 calves, 3 (A, B and C), aged 4, 8 and 4.5 months respectively, were used for sequential sampling and to follow the progression of clinical signs; the other calf (D), the progeny of heterozygous parents (at CSIRO Division of Tropical Animal Production, Rockhampton), was identified by biochemical means as being affected with glycogenosis at 3 weeks of age and was euthanased after displaying clinical signs typical of glycogenosis at 7 months of age.

Affected calves were of both sexes, came from both intensive and extensive grazing situations, and were between 3 and 9 months of age, with the exception of 3 atypical cases (calves E, F and G) aged between 1 and 4 weeks.

Blood samples collected into EDTA were kept at 15°C for 24 h before the preparation of peripheral blood lymphocytes (PBL); other EDTA-treated blood samples were obtained for the preparation of blood smears and for haematology; serum was collected for clinical biochemistry. Blood smears were also received as field submissions from 38 clinically normal animals. Gluteal

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muscle samples were taken from 3 of the 8 animals necropsied and stored as described previously (Reichmann *et al* 1989). Tissues collected for histological examination included liver, kidney, heart, skeletal muscle, intestine and, in some instances, brain, spinal cord and eye. Calves E and G were subject to similar investigations, while for F only the head was submitted to the laboratory.

Calves A, B, C and D were sampled every 3 to 12 weeks for examinations that included the measurement of AAG activity in PBL. Urine was collected on at least 3 occasions from these animals for urinalysis and storage at -60°C before thin layer chromatography. Four gluteal muscle biopsies from calf A, 3 from B and one from calf C before necropsy were also collected.

A selection of samples, including multiple EDTA-treated blood samples for smears and PBL isolation, urine, and muscle biopsies, were taken from a control group of 23 clinically normal Brahman animals, aged between 6 months and 5 years, that included 3 obligate and 9 putative heterozygotes for generalized glycogenosis and 11 that were normal.

Analytical Methods

PBL were prepared from EDTA-treated blood by density gradient centrifugation 24 h after collection (O'Sullivan *et al* 1981) and extracted with 0.5 mol/L KC1 in 0.005 mol/L acetate buffer (pH 3.5) containing 0.2% Triton X-100. The activity of AAG, relative to that of hexosaminidase and β-galactosidase, was determined and expressed in percentage terms as described previously (Reichmann *et al* 1987). The ratio of neutral to acidic α-glucosidase in plasma was determined to exclude the presence of castanospermine (Reichmann *et al* 1987).

Blood was analyzed for haemoglobin (Hb), packed cell volume (PCV) and total red (RCC) and white cell counts (WCC). Serum was analyzed for calcium, magnesium, protein, bilirubin, creatinine and urea concentrations and activities of δ-glutamyltransferase (δ-GT), creatine kinase (CK) and aspartate aminotransferase (AST) using standard methods.

Blood smears were stained with May-Grunwald Giemsa for a differential leucocyte count and with periodic acid-Schiff (PAS) (Luna 1965) for glycogen. PAS-stained smears were examined by one operator for the presence of granules in lymphocytes.

Formalin-fixed tissues were embedded in paraffin and sections stained with haematoxylin and eosin using routine procedures. In the early stages of the investigation tissues were also stained with PAS to confirm the presence of glycogen.

Urine samples were subjected to both semi-quantitative urinalysis, using a standard test-strip and thin layer chromatography (TLC) (Humbel and Collart 1975), using a sample loading of 10 μL to 30 μL.

The concentration of glycogen and AAG activity were determined in muscle biopsies as previously described (Reichmann *et al* 1989).

Biometrical Analysis

Differences in muscle AAG activity and glycogen concentrations between genotypes were tested using one-way analysis of variance. Genotype group means were compared using the protected least significant difference procedure (Snedecor and Cochran 1967).

Results

Clinical Observations

In most cases affected calves were identified and first presented for examination just before or after weaning at around 6 months of age, most often because of failure to thrive and muscular weakness. With time hypometria of all limbs became more apparent and the animals were slower to rise from sternal recumbency. In the later stages of the disease the calves often adopted a wide-based stance, developed a concave arch of the neck and had sunken eyes and, occasionally, an apparent weakness of the tongue. Most animals examined were killed or died between 6 and 9 months of age. The 3 young calves that displayed atypical clinical signs included one that failed to suckle (calf E) and 2 (calves F and G) presumed to have congenital blindness, as determined by their behavior and the necessity for hand-rearing.

Calves A, B and C survived to between 11 and 13 months of age. In each case water, good quality feed and shelter were provided in a small paddock. Two of these calves tended to remain prostrate when first received, but with assistance their condition improved and they remained upright and relatively alert until the terminal stages of the disease. Clinical signs displayed by these animals were similar to those described in the field cases.

Necropsy Findings

With the exception of one calf (E), no significant macroscopic abnormalities were observed, although in some the skeletal muscles were considered to be pale in color. The non-suckling calf (E) had hydrocephalus.

Histopathology

Swelling and multiple, fine, cytoplasmic vacuolation were diffusely present in neurones in the brain and spinal cord. Marked vacuolation was evident in skeletal muscle fibers and in some animals a few, scattered, necrotic fibers were present. Cytoplasmic vacuolation was also observed in myocardial cells, Purkinje fibers, hepatocytes, distal tubular cells of the kidney and in

smooth muscle fibers of the gastrointestinal tract. Vacuoles were shown by PAS staining to contain glycogen. In eyes examined in 2 of the older affected calves there was mild vacuolation of the ganglion cells of the retina and of glial cells in the optic nerve. Similar lesions were present in the eyes of one of the calves (G) displaying apparent blindness, but these tissues were not available for examination from the other apparently blind calf (F). There were no lesions in the cornea or lens of either of calves F or G. Histological examination of these blind calves and the non-suckling calf (E) revealed severe cytoplasmic vacuolation of neurones in the brain stem and spinal cord.

Haematology

No consistent abnormalities were found in Hb, PCV, RCC or WCC values. In affected calves cytoplasmic vacuolation of PBL was observed. All of these cells, as well as some without vacuolation, contained PAS-positive granules. All affected calves had more PAS-positive cells and with larger granules than did clinically normal animals (Table 1). Of the 4 animals examined repeatedly, A and B showed no change in the size of granules and number of PAS-positive cells, whereas, in C and D, both granule size and number of PAS-positive cells increased with time (data not shown).

Biochemistry—Clinical

Clinical biochemistry results were normal except for elevated AST (mean ± standard deviation; 734 ± 262 IU/L) and CK (1421 ± 1595 IU/L) activities in the 57 affected calves for which samples were available for analysis. Normal ranges for these enzymes in this laboratory extend to 170 IU/L for AST and 200 IU/L for CK. AST activity was also elevated (mean 518 IU/L) in serum of each of the 3 very young affected calves (D, E and G). AST and CK activity increased with the progression of the disease in the serially sampled calves (A, B, C and D). The mean (range) of enzyme activities in these calves when initially received at this Institute and at necropsy were AST 557 (502-601) and 1230 (840-1879), respectively, and CK 402 (340-493) and 2301 (442-4031), respectively. Clinical biochemistry results were within normal limits in both putative and obligate heterozygous animals.

Urinalysis of samples from affected calves was normal, except that significant concentrations (> 50 mg/100 mL) of glucose were often detected. High molecular weight oligosaccharides were found by TLC in the urine of calves A, B, C and D, but not in clinically normal animals.

Biochemistry—Glycogenosis

In the 51 cases where blood was available for examination relative AAG activity (mean ± standard deviation) in PBL was reduced to 2.7 ± 1.4%, compared with the homozygous normal range of about 70 to 150%. Low relative activities were also recorded in calves D and G when sampled at 4 weeks of age. AAG activity remained constant in 2 calves (A and B) sampled over 7 and 5 months, respectively. The ratio of neutral to acidic α-glucosidase in plasma from affected and clinically normal animals was always normal, indicating that the consumption of *Castanospermum australe* was not a complicating factor (Reichmann *et al* 1987).

In muscle mean glycogen concentration was higher and AAG activity was significantly lower in affected than in clinically normal calves (Table 2). Muscle AAG activity in heterozygotes with PBL relative AAG activity of 40 to 65% was about half that of normal animals. Tested heterozygotes with very low PBL relative AAG activi-

Table 1. Percentage (mean ± SD) of peripheral blood lymphocytes containing PAS-positive granules in Brahman calves either affected or unaffected with generalized glycogenosis

Calves	Number of calves	% Lymphocytes		
		PAS-negative	PAS-positive	
			Small granules	Large granules
Affected	42	31 ± 12	46 ± 8	23 ± 9
Clinically normal*	61	87 ± 15	10 ± 8	3 ± 4

* Includes both generalized glycogenosis-free calves from diagnostic submissions and a group of 23 clinically normal animals that included 3 obligate and 9 putative heterozygotes.

Table 2. Mean acidic α-glucosidase (AAG) activity and glycogen concentration in muscle from normal, heterozygous and generalized glycogenosis-affected calves

Genotype*	Number of animals	Muscle AAG activity ([IU/g protein] x 100)	Muscle glycogen (%wet weight)
Normal	6 (for AAG) 5 (for glycogen)	24.7 a†	1.40 b
Heterozygous with PBL relative AAG activities of 40-65%	7‡	12.9 b	1.10 b
Heterozygous with PBL relative AAG activities of < 20%	4	7.3 c	1.40 b
Affected	6	2.2 d	3.67 a
Average LSD (P = 0.05)		3.6	0.89

* Genotype defined by PBL AAG activity

† This group includes 5 obligate carrier animals

‡ F-test in the ANOVA was significant within columns; means not followed by common letters are significantly different at the 5% level. An average LSD is presented but exact values were used for pairwise testing of means.

ties (< 20%) had even lower muscle AAG activities. There was a highly significant correlation (r= 0.920) between muscle and PBL AAG activities for the 23 affected, heterozygous and normal calves for which both analyses were performed. In the 2 affected calves (A and B) serially sampled over a number of months, muscle AAG activity did not vary during this period. The glycogen concentration did fluctuate, but was lowest at the initial sampling.

Discussion

The course of the clinical disease, life expectancy and necropsy findings were similar in almost all of the cases examined. This indicated that, in the majority of Brahman cattle, generalized glycogenosis probably occurs as a single clinical entity. Of the 3 exceptions, one calf had a coincidental condition (hydrocephalus), while in the other 2 it could be argued that the blindness was not directly related to generalized glycogenosis. In contrast, generalized glycogenosis presents as more than one clinical syndrome in beef Shorthorn cattle (Howell *et al* 1981) and man (Engle *et al* 1973), with the phenotypic distinction being broadly related to the severity of the disease. Because onset of the disease precedes puberty and life expectancy is usually less than 12 months, it would appear that the disease in Brahmans more closely resembles the juvenile form in man.

Diagnoses of generalized glycogenosis can be confirmed by demonstration of a severe deficiency of AAG in PBL or skeletal muscle. In all the cases examined in this study the residual AAG activities in PBL were extremely low and provided unequivocal evidence of the existence of glycogenosis. In man, however, there is evidence of variation in AAG activity and clinical expression of the disease that is a consequence of the existence of a number of different mutations (McKusick 1990). Muscle AAG activity was markedly reduced throughout the course of the disease, yet its residual value (about 10% of normal) was greater than in other tissues (Reichmann *et al* 1989). While there is an equivalent deficiency of muscle AAG in beef Shorthorn animals with either infantile or late onset forms of the disease (Howell *et al* 1981), there have been reports in humans (Mehler and Dimauro 1977) of significant amounts of residual enzyme that typically were associated with the less severe, adult form of the disease. Howell *et al* (1984) were able to distinguish between heterozygous and normal genotypes using enzyme analysis on muscle biopsies. A similar differentiation occurred with the Brahman genotypes in this study, with AAG activity from both muscle and PBL being highly correlated. Even though PBL AAG activity is used in a control program to identify heterozygous stud Brahman animals (Reichmann *et al* 1987), either PBL or muscle could, theoretically, be reliably used to predict genotype.

We found no evidence to suggest that castanospermine was a factor contributing to low relative AAG activities (7%-20%) in PBL from 4 clinically normal, putative heterozygotes included in this study, or in other Brahmans tested elsewhere (Reichmann, unpublished). Low relative AAG activities in the progeny from these same animals (Reichmann, unpublished) suggests that they may have a genetic basis. Healy *et al* (1987) observed similarly low relative AAG activities in PBL from 2 bulls, one presumptive and the other an obligate heterozygote. The occurrence of such low activities in phenotypically normal animals raises the possibility of the existence of heterogeneity for reduced AAG activity in Brahmans and may account for the apparent non-autosomal recessive inheritance of genotype diagnosed in progeny of these 2 bulls (Healy *et al* 1987). In humans, the presence of 2 alleles, whose effects upon AAG activity vary, has been detected within the one family (Koster *et al* 1978).

Our observations suggest that examination of PAS-stained blood smears will be a useful field test in diagnosing generalized glycogenosis. Glycogen, as PAS-positive granules, some of which were associated with cytoplasmic vacuoles, was found consistently in a high proportion of lymphocytes from affected calves. This rapid, non-invasive method using blood smears has advantages, particularly if a quick diagnosis can provide sufficient time to identify the obligate carrier dam of a calf in the field before weaning. The demonstration of glycogen-filled lysosomes in lymphocytes has been previously proposed as a simple means of diagnosing generalized glycogenosis in humans (Von Bassewitz *et al* 1977), but the technique depends on electron microscopy. Cytoplasmic vacuolation of lymphocytes occurs with both inherited and swainsonine-induced deficiency of α-mannosidosis (Huxtable and Dorling 1982). However, the neurological dysfunction that is a major feature of α-mannosidase deficiency readily distinguishes it from generalized glycogenosis. Likewise, *Swainsona* intoxication can affect animals of all ages and breeds and there is usually evidence of consumption of the plant.

Glycogen was shown to accumulate in the muscle of affected calves but not in heterozygous animals, including those with very low PBL AAG activities. Presumably, in these cases there was sufficient enzyme activity to metabolize intralysosomal glycogen. Similar results were recorded by Howell *et al* (1981) for heterozygous and affected beef Shorthorn animals, but they did observe ultrastructural evidence of excess cytoplasmic glycogen deposited in some muscle cells and fibroblasts of heterozygous animals.

Although lacking specificity, findings of persistently elevated AST and CK activities in serum of calves that develop ill-thrift around weaning would justify ap-

plication of more specific diagnostic aids. Evidence from field cases and serial sampling of affected calves indicated that these enzyme elevations were greater during the terminal stages of the disease and were not associated with recumbency. As suggested by Edwards and Richards (1979), the elevation in AST probably reflects continuing mild skeletal muscle damage. Evidence of progressive muscle fiber damage in beef Shorthorn animals has been documented by Howell *et al* (1984). In contrast, CK, which has a short half-life compared with AST, is generally not grossly elevated. Increased enzyme activities in very young affected calves suggest they are born with a degree of muscle fiber damage.

We have shown that TLC analysis of oligosaccharides in urine is also a useful laboratory aid to confirm cases of generalized glycogenosis. The presence of excess amounts of oligosaccharides in the urine of calves with generalized glycogenosis parallels that in humans (Blom *et al* 1983). Hallgren *et al* (1974) found oligosaccharides, as well as identifying a tetrasaccharide, in the urine of a patient suffering the juvenile form of this disease.

Our observations on field cases and affected calves retained at this Institute suggest that the progression of the disease and life expectancy were influenced by age at weaning and exacerbated by extensive grazing conditions. The clinical signs displayed by affected calves were often unremarkable, but typically reflected progressive muscular weakness. We have shown, however, that life expectancy can be extended up to 13 months of age by the provision of quality feed, water and shelter.

The histological changes we observed were similar to those reported by O'Sullivan *et al* (1981) in Brahman calves, and by Cook *et al* (1982) in Shorthorns. It is probable that in the non-suckling calf with hydrocephalus generalized glycogenosis was a coincidental disease. Except for 2 cases in Beef Shorthorns reported by Cook *et al* (1982) involving only slight dilation of lateral ventricles, hydrocephalus is not apparently a feature of generalized glycogenosis as it is with mannosidosis (Jolly and Thompson 1978). Based on previous diagnostic enquiries we have found no relationship between AAG activity, vacuolation of neurones and the incidence of a non-suckling condition in very young Brahman calves. No cause for the blindness in 2 calves was detected, although it was not considered due to generalized glycogenosis because the histological changes in the optic nerve and retina from the one calf available for study were only mild and similar to changes in 2 other affected Brahman calves that were not blind. Furthermore, Cook *et al* (1982) and O'Sullivan *et al* (1981) also described retinal changes in cases of glycogenosis that were not accompanied by loss of sight.

The biochemical demonstration of AAG deficiency and/or histological evidence of tissue vacuoles containing glycogen stainable with PAS provided a definitive diagnosis of generalized glycogenosis in most cases investigated. In the remainder a presumptive diagnosis was made, based on vacuolation of cells in tissues such as brain and skeletal and cardiac muscle in young Brahman-type calves displaying typical clinical signs. PAS staining was not always undertaken because a presumptive diagnosis was regarded as being sufficient. Furthermore, histological examination of glycogen in tissues by PAS staining can require particular sample preservation and preparation (Bancroft and Cook 1984).

Over the last 11 years 96 Brahman calves were diagnosed with generalized glycogenosis. The actual incidence in Queensland is thought to be much higher than these figures indicate. Brahman animals commonly are extensively grazed and, since anecdotal evidence from producers indicates that many calves die in the field, often through misadventure, or are culled if suffering from ill-thrift, it is likely that a significant number of cases are undiagnosed. The prevalence of this disease is expected to diminish following the introduction of a voluntary control program to identify heterozygotes in Brahman studs (Reichmann *et al* 1987).

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The effect of dietary urea on the superovulatory response of beef cattle.

M. Monaci, U. Chicchini, O. Olivieri, P. L. Casey, W. G. Rankin, and R. A. Godke.
Atti SISVet, (1989) XLIII, 413.

The objective of this experiment was to evaluate the effect of dietary urea on superovulatory response of cattle. The animals in Trt. A and B were maintained on pasture and those in Trt. B given 100g of urea in 1Kg of mixed feed daily/animal for 21 days prior to and during superovulation through embryo collection. Embryos were morphologically evaluated (grade 1 to 4). BUN was monitored weekly (mg/100ml). The mean BUN values ranged in Trt. A from 6.2 to 7.5 while in Trt. B from 7.3 to 19.5. Mean follicles/donor was 2.8 and 3.5 for Trt. A and B; mean corpora lutea/donor was 7.3 and 5.5 for Trt. A and B. Mean n. viable embryos/donor in Trt. A was 3.0 and 1.3 for Trt. B, while mean n. of grade-4 embryos and UFO/donor was 1.0 and 0.16 in Trt. A, 0.88 and 0.88 in Trt. B respectively.

Diaplacental infections with ruminant pestiviruses.

B. I. Osburn and G. Castucci.
Arch. Virol., (1991), suppl. 3, 71-78.

Pestiviruses are capable of causing diaplacental infections. Maternal viremias are important for localizing virus in the ruminant placentome. Placental lesions occur with cytopathic BVDV and noncytopathic BVD. The ruminant fetus is very susceptible to pestivirus infections once the virus crosses the placenta because the fetus is 1) agammaglobulinemic, 2) immunologically immature, and 3) it has many immature organ systems with undifferentiated cells. Cytopathic BVDV (NADL) in calves and noncytopathic BVD (BD-31) in lambs cause a variety of clinical syndromes including early embryonic death, abortion, stillbirth, malformed fetuses, and/or low birth weight with viral persistence and immunological tolerance. The cytopathic BVDV (NADL) reviewed herein caused pulmonary, placental and dermal lesions when infection occurred at 80-90 days gestation. In contrast, infection at 140-150 days resulted in retinal dysplasia and cerebellar hypoplasia. The lesions were attributed to direct viral cytopathology. Noncytopathic BVD (BD-31) in lambs, with hairy fleece caused tonic-clonic tremors. The lambs were of low birth weight, persistently viremic and immunologically tolerant. The lambs were hypothyroid and had a severe hypomyelination. It is hypothesized that the central lesion leading to many of the neural, skeletal and dermal lesions was the endocrine dysfunction leading to hypothyroidism.